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Development of SYN-004, an oral beta-lactamase treatment to protect the gut microbiome from antibiotic-mediated damage and prevent *Clostridium difficile* infection

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ABSTRACT

The gut microbiome, composed of the microflora that inhabit the gastrointestinal tract and their genomes, make up a complex ecosystem that can be disrupted by antibiotic use. The ensuing dysbiosis is conducive to the emergence of opportunistic pathogens such as *Clostridium difficile*. A novel approach to protect the microbiome from antibiotic-mediated dysbiosis is the use of beta-lactamase enzymes to degrade residual antibiotics in the gastrointestinal tract before the microflora are harmed. Here we present the preclinical development and early clinical studies of the beta-lactamase enzymes, P3A, currently referred to as SYN-004, and its precursor, P1A. Both P1A and SYN-004 were designed as orally-delivered, non-systemically available therapeutics for use with intravenous beta-lactam antibiotics. SYN-004 was engineered from P1A, a beta-lactamase isolated from *Bacillus licheniformis*, to broaden its antibiotic degradation profile. SYN-004 efficiently hydrolyses penicillins and cephalosporins, the most widely used IV beta-lactam antibiotics. In animal studies, SYN-004 degraded ceftriaxone in the GI tract of dogs and protected the microbiome of pigs from ceftriaxone-induced changes. Phase I clinical studies demonstrated SYN-004 safety and tolerability. Phase 2 studies are in progress to assess the utility of SYN-004 for the prevention of antibiotic-associated diarrhea and *Clostridium difficile* disease.

1. Introduction

The gut microflora and their collective genomes, referred to as the microbiome, play a key role in health and disease. The gut microbiome works symbiotically with the body to aid digestion, facilitate the absorption of nutrients, synthesize vitamins, block the invasion of pathogenic bacteria across the intestinal mucosal barrier, and provide protection against virulent organisms [1,2]. Recent evidence has demonstrated that the influence of the gut microbiome is far reaching, affecting immune, metabolic, cardiovascular, and neurological functions [3]. Alterations or imbalances in the ecology of the gut, termed dysbiosis, have been linked to a variety of disease states including obesity, allergies, asthma, autism, and a rapidly growing list of other conditions [3–5]. In fact, the gut microbiome functions like an organ [3], and our understanding of its role in human physiology is still evolving.

Anything that disrupts the normal ecology of the gut microbiome can have a negative impact on human health. While antibiotics are life-saving, their use is associated with dysbiosis in the GI tract, which can increase susceptibility to pathogens such as *Clostridium difficile* [4,6,7] and lead to overgrowth of resistant bacteria [8–10]. Beta-lactams are the most commonly used

Abbreviations: AAD, antibiotic-associated diarrhea; CDI, *Clostridium difficile* infection; Conc, concentration; C_{max}, maximum concentration; CRO, ceftriaxone; FMT, fecal microbiota transplantation; GI, gastrointestinal; IV, intravenous; MIC, minimal inhibitory concentration; spp, species.

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intravenous (IV) broad spectrum antibiotics, accounting for 72% of IV antibiotic use in the United States annually [11]. The most frequently prescribed beta-lactams are piperacillin, including piperacillin/tazobactam, and ceftriaxone, both of which are associated with an increased risk of *C. difficile* infection (CDI), particularly the latter [12].

Current treatments for CDI remain relatively limited, with metronidazole and vancomycin still the cornerstones of therapy. Antibiotic-mediated treatment of CDI is characterized by high rates of recurrence [13]. Recognition of the importance of the gut microbiome in prevention of CDI has prompted investigation into therapies aimed at restoring the normal microbial balance, such as the use of probiotics [3] and fecal microbiota transplantation (FMT) [14,15]. FMT reconstitutes the gut microflora with organisms native to the fecal donor and therefore does not necessarily re-establish the patient's pre-disease microbiome. As it is becoming increasingly apparent that host genetics influence the structure of the microbiome, and that the gut microbiome affects many, if not all organ systems, the long-term outcomes of FMT must be carefully evaluated [14,15]. While each of these interventions provides treatment for CDI once the infection is established, prevention of antibiotic-associated dysbiosis may represent a complementary strategy to preclude the occurrence of CDI.

One novel strategy to protect the microbiome from antibiotic-mediated dysbiosis and to prevent CDI is the use of beta-lactamase enzymes to degrade the antibiotics in the GI tract.

1.1. Beta-lactamases: from enemies to therapies

Beta-lactamases are natural enzymes that degrade beta-lactam antibiotics, confer antibiotic resistance, and dramatically complicate the treatment of bacterial infections. Thus, they are rightfully considered the "enemy", and substantial efforts have been directed towards their characterization and inhibition [16]. However, beta-lactamases can also be harnessed as therapeutics to protect the "good", commensal gut microflora from unwanted damage incurred by antibiotic use. In 1999, Ipsat Therapies, OY (Helsinki, Finland) had the foresight to recognize that an orally administered beta-lactamase had the potential to degrade residual antibiotics in the proximal GI tract, before they could adversely affect the colonic microflora. The ensuing beta-lactamase treatment, termed P1A, was designed to be used in conjunction with parenterally administered beta-lactam antibiotics, which are secreted through the biliary system into the proximal small intestine.

P1A is a naturally-occurring penicillinase isolated from *Bacillus licheniformis* (the PenP protein) [17]. It was manufactured in *Bacillus subtilis* and formulated into pellets with a Eudragit®-based, acid-resistant coating for oral delivery [18]. Multiple animal and human studies demonstrated that P1A was released in the proximal GI tract and effectively degraded residual penicillins [18,19]. In mice, P1A prevented colonization by pathogenic organisms including *C. difficile*, vancomycin-resistant *Enterococcus faecium* (VRE), *Klebsiella pneumoniae* and *Candida glabrata* [20,21]. In dogs, P1A efficiently degraded residual ampicillin in the intestinal fluid, chyme, while having no effect on serum ampicillin levels [22]. Additionally, P1A protected the gut microbiome from antibiotic-mediated disruption [19,23] and prevented the selection of ampicillin-resistant bacteria [18,24].

Based on these promising preclinical efficacy data, P1A was subsequently evaluated in four successful Phase 1 and 2 clinical trials conducted in Europe [18,19]. In healthy human subjects and hospitalized patients with upper respiratory tract infections treated with IV ampicillin, P1A preserved microbiome diversity and prevented the emergence of ampicillin-resistant coliforms [18]. Similarly, in healthy volunteers treated with IV piperacillin/tazobactam, P1A effectively degraded pipercillin in the GI tract, preserved the microbiome, and prevented the occurrence of antibiotic-associated diarrhea [19]. Importantly, P1A was not detected in the plasma of treated subjects and had no effect on plasma levels of pipercillin [19]. Taken together, these data demonstrate the clinical utility of this novel therapeutic approach in protecting the microbiome from antibiotic-mediated damage without affecting systemic antibiotic efficacy. However, as a penicillinase, the clinical utility of P1A was limited. P1A did not efficiently degrade most cephalosporins including ceftriaxone, which is the most frequently used IV beta-lactam antibiotic [11] and is associated with a high risk for CDI [12]. In 2012, Synthetic Biologics, Inc. acquired the beta-lactamase technology, including a second generation product candidate, P3A. Now referred to as SYN-004, P3A was engineered from P1A with the intent of broadening its antibiotic degradation profile to include cephalosporins. SYN-004 is currently undergoing Phase 2 clinical analyses [25]. Here, we report the data supporting the clinical application of SYN-004.

2. Methods

2.1. Production of beta-lactamases

P1A was manufactured in *B. subtilis* as described [22]. SYN-004, a protein of 29 kDa, was manufactured in *Escherichia coli*. SYN-004 was formulated for oral delivery by incorporation into Eudragit®-coated sucrose pellets designed to release active enzyme at pH 5.5 or greater [26]. The pellets contained approximately 15% SYN-004 [26]. Gelatin capsules suitable for oral delivery were filled with the pellets for a total SYN-004 content of 75 mg/capsule.

2.2. In vitro antibiotic degradation analyses

The antibiotic degradation kinetics of P1A and SYN-004 enzymes were determined for ampicillin, amoxicillin, piperacillin, ceftriaxone, cefazolin, cefuroxime, cefoperazone, and cefotaxime. The steady-state K_m and k_{cat} values for each antibiotic were determined by measuring substrate hydrolysis under initial rate conditions with Hanes linearization [27] of the Michaelis-Menten equation. The reactions were performed in 20 mM phosphate buffer (pH 7.0) at 30 °C. Briefly, dilutions of each antibiotic were prepared in sodium phosphate buffer and distributed to individual wells of a 96-well plate. The plate was pre-incubated at 30 °C for 5 min, after which P1A (1.0 nM) or SYN-004 (1.0 nM) was added to the plate. The absorbance at 235 nm (ampicillin and piperacillin), 242 nm (amoxicillin), 257 nm (ceftriaxone), and 264 nm (cefazolin, cefuroxime, cefoperazone, cefotaxime) of the individual wells was determined every 5–8 s over a 10–20 min period of incubation. The initial velocity was calculated as the change in absorbance per min (mU_{abs}/min) of the reaction as determined using SoftMax Pro 5.4 software from the slope of the curve within the linear range of the reaction. Following the kinetic analysis, an endpoint reading was taken to determine the pathlength (cm) of the specimen in each individual well. The velocity (mU_{abs}/min) values were normalized to a 1 cm pathlength by dividing the values by the measured pathlength. The normalized velocity values (mU_{abs}/min-cm) were then converted to velocity (nmole/sec-cm) using an experimentally determined extinction coefficient specific for each individual antibiotic at the given wavelength. The data were imported into Prism GraphPad 5 for determination of Michaelis-Menten kinetics by non-linear regression. The analysis was performed a minimum of 3 times for each antibiotic substrate. Kinetic data (K_m, k_{cat}, and k_{cat}/K_m) were calculated from the mean of the experiments. Data are presented as the mean ± standard deviation.

The antibiotic inactivation activities of P1A and SYN-004 were